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Award Number: W81XWH-08-1-0148

TITLE: Acute Lung Injury: Making the Injured Lung Perform Better and Rebuilding Healthy Lungs

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REPORT DATE: July 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2011	Annual	1 July 2010 – 30 June 2011

4. TITLE AND SUBTITLE

Acute Lung Injury: Making the Injured Lung Perform Better and Rebuilding Healthy Lungs

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0148

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Boston Medical Center
Boston, MA 02118

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Acute lung injury (ALI) is a complex syndrome characterized by diffuse injury to the alveolar epithelial surface resulting in a marked impairment in the ability to oxygenate blood. The goals of our application are to facilitate the rational development of ventilatory and cell based strategies to treat the ALI. As we have previously noted, ALI is associated with severe infections, exposure to toxins, trauma, and multiple blood transfusions; all of these conditions afflict patients with cancer. This is compounded by the fact that cancer patients are vulnerable to development of ALI as a result of the immunosuppressive effects of chemotherapy and the debilitating effects of cancer. It is also important to note that active military personnel are also at risk for ALI because of battle induced trauma and the consequent need for blood transfusions. In Project 1, we proposed to develop a new mode of ventilation (variable ventilation) that will minimize the toxic effects of conventional mechanical ventilation in patients with ALI. In the past year, we resolved all technical and engineering barriers, and have developed a fully functional variable ventilator (running on a Covidien Puritan-Bennet 840 ventilator platform). We completed in-laboratory testing to confirm the correct function and alarm functionalities of the system. We are in the final phases of now completing a FDA Investigational Device Exemption (IDE) submission, which we plan to submit to FDA this month. Once received, we will submit the final protocol to the Department of Defense Human Research Protection Office (HRPO) for approval. In support of this goal, we monitored the clinical characteristics of the patients with respiratory failure in our intensive care units so that we can develop the optimal recruitment strategy for an informative and effective clinical trial once final FDA IDE and DoD HRPO approvals are in hand. In the preclinical Project 2 study in mice, we have continued to be focused on optimizing the derivation of progenitor cells that can be administered to mice with ALI with the intent of reconstituting the damaged gas exchange surface. In the past year, we generated a specific new mouse that has facilitated the derivation of these cellular progenitors. Using this mouse, we established conditions that permit the derivation of highly specific lung epithelial derivatives. To summarize in the past year, we have: 1) resolved technical and logical issues, are preparing an IND, and are poised to begin a clinical study 2) and continued to optimize derivation of mouse lung epithelial progenitor cells for testing in mice with ALI.

15. SUBJECT TERMS
Lung injury, cancer, ventilator, stem cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

The overall goal of our application is to develop strategies to treat the acute lung injury (ALI) syndromes that complicate cancer care. As we have discussed in previous reports, ALI occurs in a variety of clinical and military settings. These include: severe infections, exposure to toxins, trauma, multiple blood transfusions, and from the untoward side effects of chemotherapy. While the pathology of ALI is very complex, it is predominantly manifested by signs of diffuse injury to the alveolar wall and epithelial gas exchange surface. This includes massive infiltration of inflammatory cells into distal lung regions, loss of epithelial and endothelial integrity, and marked edema. Together, these effects serve to inhibit gas exchange; hence the difficulty in oxygenating patients with ALI. In this context, it has been known for many years that disruption of type I cells, which comprise the vast majority of the gas exchange surface, is the major site of cellular injury. To meet our objectives, we proposed 2 Projects. In Project 1, we planned to extend studies performed in animals and to extend observations in humans to determine whether we could design a more efficacious and safe method for mechanically ventilating patients with ALI. This objective derives from the fact that current modes of ventilation, *by themselves*, worsen ALI by directly causing a stretch-induced injury to the distal lung and by amplifying inflammation. Specifically, we proposed to evaluate the efficacy of so-called *variable ventilation* in patients with ALI relative to conventional modes of ventilation. The ultimate goal of this Project is a small clinical trial in patients with ALI to directly compare these types of ventilatory support. In the past year, we have resolved a variety of technical, logistical, and safety issues in anticipation of clinical study that will be performed in the upcoming year. In Project 2 of this proposal, we proposed to assess whether we could directly enhance the healing of the injured type I cell population by administering cellular precursors to type I cells. A major objective is was to identify the optimal type I cell precursor population that is scalable and can sustain rational clinical interventions. As previously discussed, a major part of this strategy is the development of a cell therapy that is based on the use of embryonic stem cells, which are and are not skewed towards lung epithelial differentiation. This is a pre-clinical study that uses laboratory mice, mouse cells, and a well-described model of chemotherapy induced ALI. In this report, I will discuss the progress made in each of these 2 Projects.

Body of Progress Report

Below is a summary of the progress and achievements for the 2 Projects that comprised the original parent proposal.

Project 1: We will determine if variable ventilation is a more effective mode of ventilation in patients with ALI.

Our clinical study of variable ventilation in acute respiratory failure continues to make progress toward to reaching the point at which we can begin subject recruitment for a small clinical study. Over the past year, we have finally resolved all technical and engineering barriers, and we now have developed a fully functional variable ventilator that runs on a *Covidien Puritan-Bennet 840* ventilator platform. In the past 2 months we have completed in-laboratory testing to confirm the correct function and alarm functionalities of the system. We are now completing the preparation of the FDA Investigational Device Exemption (IDE) submission, which we plan to submit to the FDA this month. Once we have received the FDA IDE, we will submit the final protocol details to the Department of Defense Human Research Protection Office (HRPO) for approval. Simultaneously with this technical work, we have monitored the clinical characteristics of the patients with respiratory failure in our intensive care units so that we can develop the optimal recruitment strategy for recruiting patients into our variable ventilation trial once final FDA IDE and DoD HRPO approvals are in hand.

Simultaneously with our work to develop and conduct a trial of variable ventilation in humans, we carried out theoretical modeling to provide further insights into the likely impact of variable ventilation (VV). Since the stretch pattern during VV has been shown to increase surfactant release both in animals and cell culture, we have hypothesized that there are combinations of PEEP and V_T during VV that lead to improved alveolar recruitment compared to conventional mechanical ventilation (CV). To test this hypothesis, we developed a computational model of stretch-induced surfactant production and release combined with abnormal alveolar mechanics of the injured lung during mechanical ventilation. Using this model, we compared alveolar recruitment-de-recruitment behavior over a wide range of PEEP and V_T during both VV and CV. The model developed in this study demonstrates the significance of the cellular stretch-induced surfactant release relationship with respect to whole lung stability. Maintaining epithelial cell stretch above a critical threshold with either PEEP or V_T may help stabilize the injured lung (see Figure 1, next page). Moreover the injured lung can see additional benefit from breath-by-breath variation of tidal volumes which maintains the lung periphery open under a dynamic equilibrium with a better outcome than the corresponding conventional ventilation strategy. Thus, irrespective of the particular model used in this study, our results point to the clinical significance of ventilator-patient coupling. A manuscript detailing these findings is ready for submission Most importantly the implications of these favorable findings will be tested in our clinical study.

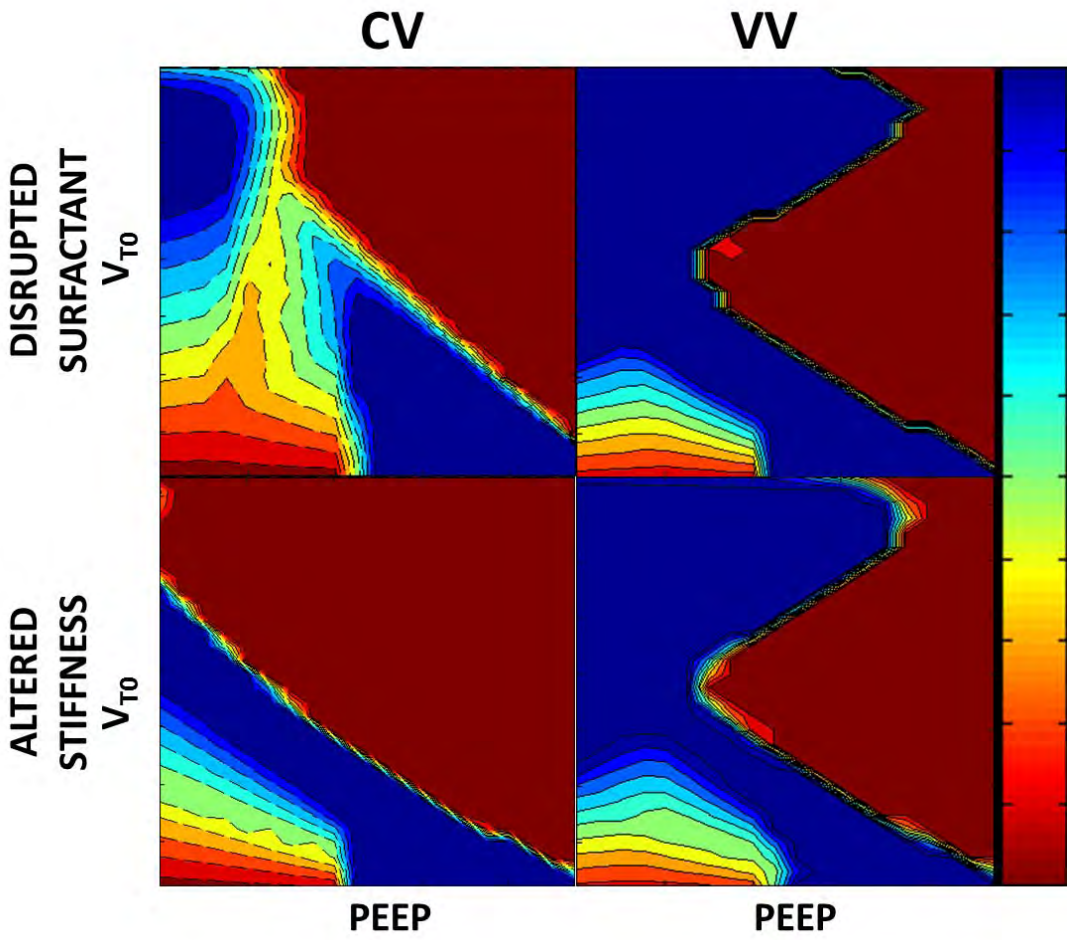

Figure 1: Computational model of stretch-induced surfactant production and release. Computer Simulations allowed us to compare and contrast the results for applying Variable Ventilation (VV) or Conventional Ventilation (CV) for heterogeneous surfactant release as well as heterogeneous alveolar stiffness. The following contour plots represent the fraction of the lung which are neither collapsed nor overdistended when ventilating with a given PEEP and V_T for CV and VV. For a disruption of normal homogeneous surfactant release under CV (top left panel), alveolar collapse occurs at low-PEEP-low- V_T settings, while overdistention occurs for high-PEEP-high- V_T settings resulting in two relatively small disjoint regions (blue) whereby alveolar injury is prevented. When VV is applied (top right panel), one contiguous region of PEEP and V_T allows for full recruitment without overdistention. Overall, the possible combinations of PEEP and V_T during VV are much greater than during CV. For the heterogeneous alveolar stiffness case under CV (bottom left panel), again alveolar collapse occurs at low-PEEP-low- V_T settings, while overdistention occurs for high-PEEP-high- V_T settings. However, in this case a contiguous band of desirable PEEP-V_T settings exist. When VV is applied (right panel), the size of the desirable region is significantly larger.

Project 2: We will establish a pre-clinical program conducted in laboratory mice with the objective of developing cell-based treatments for ALI.

As in previous years, our goal is to develop an autologous cell-based therapy to reconstitute the injured lung epithelium. A key element of this work is to evaluate and identify the optimal exogenous progenitor cell population with lung epithelial reparative properties. As we discussed in last year's report, we propose to include a newly discovered type of pluripotent stem cell, termed iPS cells, in addition to the originally proposed studies utilizing embryonic stem (ES) cells. Several practical advantages of these cells include the fact that these cells have a high degree of plasticity, are easily derived, highly scalable, and since they can be readily derived from individual mice (or humans) they thereby circumvent immune and ethical issues. Indeed these iPS cells have been shown to serve as precursors for a variety of differentiated cell types. One of our goals is to develop systems and conditions to assess how we can enhance the differentiation of these cells in lung epithelial progenitors and into type I cells themselves. To meet theses goals we generated a TTF1-GFP knockin reporter ES cell line and mouse. Notably, TTF-1 (also known as NKX2.1) is a transcription factor required for development of the lung endodermal component. With GFP expressed from the same locus, GFP expression in iPS or embryonic cells derived from this mouse can serve as a marker of lung differentiation. Once in vitro conditions of differentiation are established, we can then administer lung endodermal or fully differentiated type I cells that we derive for their clinical benefits in bleomycin-injured mice. This approach is consistent with our original objectives set forth in this proposal.

This year we completed a TTF1-GFP knockin ES cell line which was used to generate a knockin mouse line after transplantation of the ES cells into a blastocyst, followed by implantation in a pseudopregnant foster mother. The engineered ES cells were transmitted through the germ line to establish this unique TTF1-GFP knockin mouse line. We have characterized the adult mouse and found the GFP reporter to be faithful and specific as the GFP reporter gene is expressed in the lung epithelium, thyroid gland, and in the forebrain, reflecting the localization of endogenous TTF1 mRNA and protein expression. Derivation of this mouse line is shown in Figure 2 (next page). We are currently focused on characterizing the developmental expression kinetics of the GFP reporter in this mouse during early lung, thyroid and brain development.

Our derivation of a unique novel tool, the TTF1-GFP knockin reporter ES cell line and mouse allows for the first time the purification and study of the first primordial lung progenitors specified during development. This is a significant accomplishment as virtually nothing is known about these primordial and broadly multipotent lung epithelial progenitors

(see Figure 3, page 9). We can now focus on delineating the genetic and epigenetic global program of the primordial TTF1-GFP+ primordial lung progenitors. We are particularly focused now on studying in detail the epigenetic state of the TTF1 locus, including DNA methylation state, histone methylation state, and chromatin remodeling factors that control the state of expression of this locus. This will provide insight into the factors controlling lung epithelial differentiation. We will soon publish our new TTF1-GFP reporter ES cell line as well as the protocol that allows the lung endodermal directed differentiation of this cell line. Soon we will be injecting these lung epithelial precursors into injured mice. Overall our results have considerable potential for the development of a cell-based therapy for ALI and for the field of lung regenerative medicine in general.

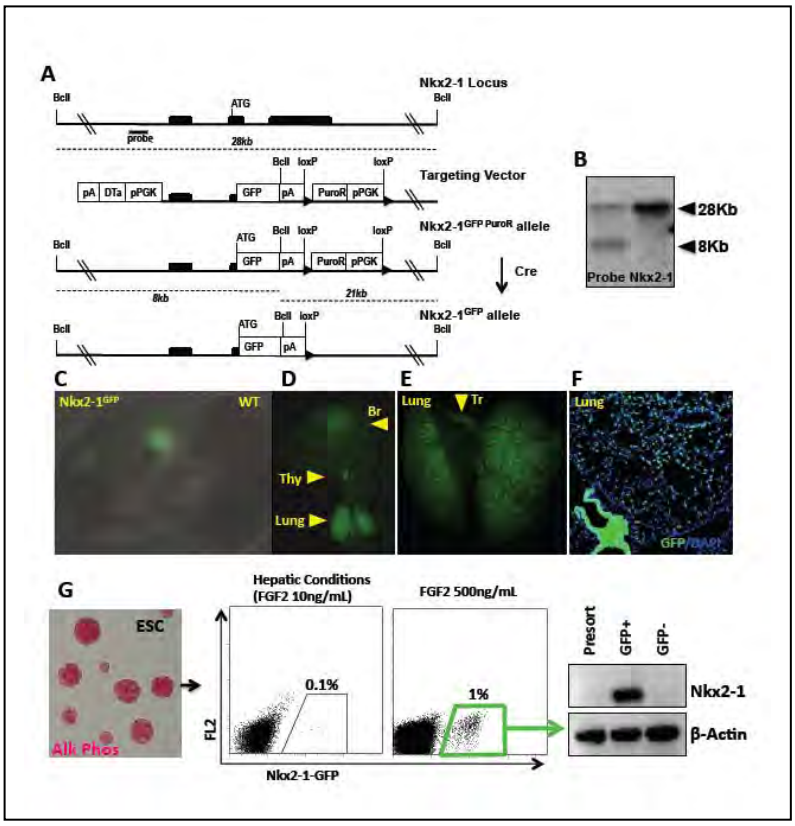

Figure 2: Gene targeting of the Nkx2-1 locus by homologous recombination generates an Nkx2-1GFP knock-in reporter ES cell line and mouse. A: Schematic of the mouse Nkx2-1 locus, showing 3 exons (black bars) and the most common ATG start site. The targeting vector introduces the GFP reporter gene in place of the native Nkx2-1 ATG start site along with an additional Bcl I restriction site and a floxed PGK-driven puromycin resistance cassette, which was excised upon transient Cre-exposure. B: Southern blot of Bcl I-digested gDNA from the targeted clone (left lane) and the parental, untargeted mouse ES cell line (right lane), demonstrating untargeted (28kb) and targeted (8kb) alleles. (C-E) Characterization of a heterozygous Nkx2-1GFP knock-in mouse embryo (E14.5) generated after germline transmission of the ES cell line shown in B and G. C and D: GFP fluorescence in the lung, thyroid (thy), and forebrain (Br) regions of the knock-in embryo vs. a wildtype (WT) littermate. In the E14.5 lung whole mount (E) GFP fluorescence appears in an epithelial pattern, including expression in the trachea (Tr; arrowhead). (F): Frozen tissue section from a post-natal lung reveals GFP fluorescence in airway epithelia and alveolar epithelial type 2 cells. (G) Flow cytometry sorting based on GFP fluorescence of ES-derived cells on day 14 of differentiation after Activin stimulation until day 5 followed by the indicated endodermal media from day 5-14. RT-PCR analysis shows expression of the indicated mRNA in each sorted population. Photomicrograph shows the morphology of the starting (day 0) undifferentiated ES cell colonies, maintained in serum-free, feeder-free conditions and stained for alkaline phosphatase, a conventional marker used to confirm the undifferentiated state of the starting cell preparation.

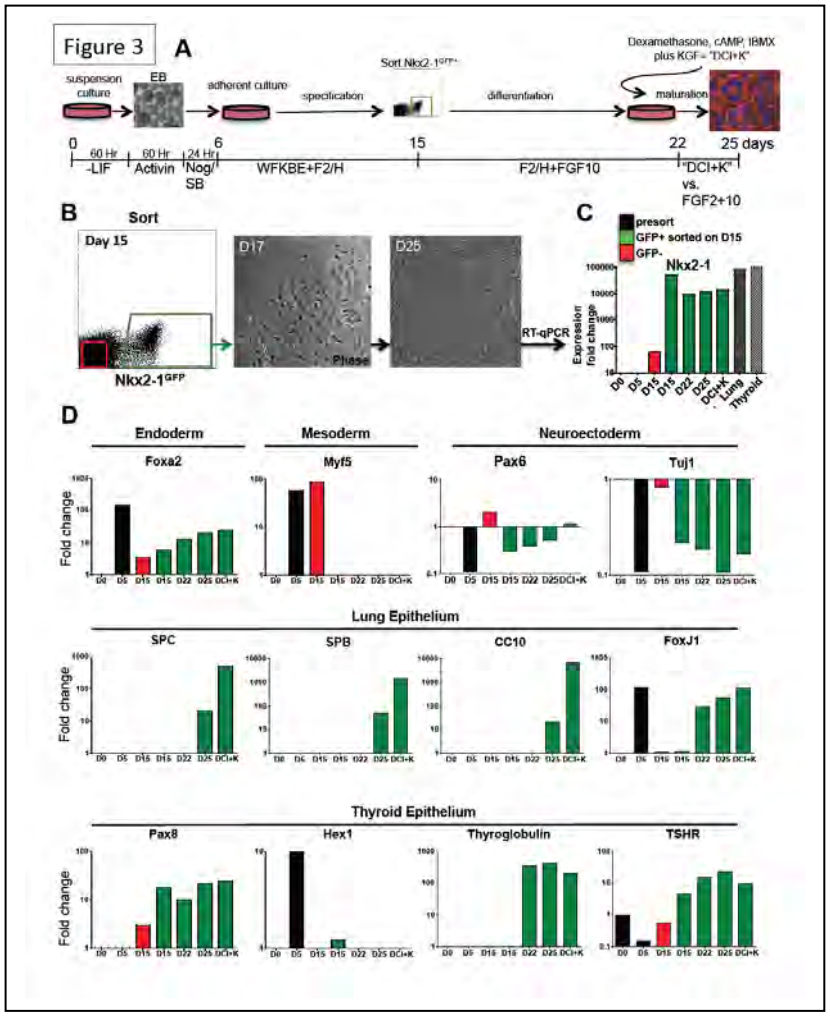

Figure 3: Purified endodermal Nkx2-1^{GFP+} progenitors derived from ES cells can proliferate in culture and express a repertoire of lung and thyroid lineage genes. (A) Schematic of 25 day culture protocol for directed differentiation of ES cells into Nkx2-1^{GFP+} (B) Sort gate used to purify D15 GFP+ cells for replating and outgrowth is indicated. Sorted, replated cells were imaged on days 17 and 25. (C) Expression of Nkx2-1 mRNA (average fold change compared to ESC by quantitative RT-PCR of technical triplicates) is indicated for each population. E18.5 lung and thyroid RNA extracts served as positive controls. (D) Quantitative RT-PCR analysis of the same populations shown in B. Expression of each indicated gene is shown for each time point in a single representative experiment. Bars indicate average fold change over ESC and are shown without error bars as is appropriate for averaged technical triplicates in a single experiment. Results are representative of 4 repeated experiments. DCI+K=cells exposed on D22-25 to maturation media containing dexamethasone, cyclic AMP, IBMX, and KGF. F2/H=FGF2+Heparin. WFKBE=Wnt3a, FGF2, KGF, BMP4, and EGF. Nog/SB=Noggin+SB431542.

Key Research Accomplishments

● Resolve technical issues limiting administration of variable ventilation in humans

● Poised to finalize and submit FDA IDE application

● Monitored the clinical characteristics of the patients with respiratory failure in our intensive care units so that we can develop the optimal recruitment strategy

● Developed a computational model of stretch-induced surfactant production and release combined with abnormal alveolar mechanics of the injured lung during mechanical ventilation.

● Several manuscripts ready for submission from both Projects

● Development of TTFa-GFP knockin mouse

● Optimization of conditions for derivation of differentiated lung epithelium from iPS cells

● Manuscript on genetic differences between iPS and embryonic stem cells published

● Micro-array analysis of lung endodermal progenitors

Reportable Outcomes

1) Completed in-laboratory testing to confirm the correct alarm functionalities of variable ventilatory system

2) Resolution of technical issues limiting application of variable ventilation in humans

3) Final preparation of IND for variable ventilation

4) Successful computer modeling of variable ventilation (manuscript in preparation)

5) Initial screening of patients for clinical study.

6) Generation of TTF-1GFP knockin mouse

7) Delineation of clinical grade mouse iPS cells for from the TTF-1GFP knockin mouse

Conclusion

We have made considerable progress in both Projects of the original parent grant. For Project 1, we have resolved all the technical issues that previously limited application of variable ventilation to human patients. Once an IND is approved, we will seek final approval from the DOD and our IRB to initiate a small pilot study in patients with ALI in our ICUs. In anticipation, we have begun to evaluate the characteristics of such patients in our ICUs that may be suitable for inclusion in this study.

In Project 2, we have continued to optimize the derivation of stem cell preparations for delivery to mice with ALI. This work has been markedly facilitated by the development of a unique tool, the TTF1-GFP mouse. In the past year, we have identified conditions that enhance the differentiation of iPS cells from this mouse into lung epithelium and their precursors. These cells will be tested for their ability to heal a damaged alveolar epithelial surface in mice subjected to bleomycin-induced lung injury. As in previous years, we continue to elucidate the molecular factors that control differentiation of these cells into lung epithelium. Together, this has propelled the possibility of a cell-based therapy for ALI.